Virology **290,** 211–223 (2001)
doi:10.1006/viro.2001.1165, available online at http://www.idealibrary.com on IDEAL®

The Baculovirus Transcriptional Transactivator *ie0* Produces Multiple Products by Internal Initiation of Translation

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Received July 3, 2001; accepted August 24, 2001

Ie0 is the only gene of the baculovirus *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) that is known to be spliced. In this study, cDNAs of *ie0* were isolated, cloned, and sequenced. It was observed that IE0 contains 35 amino acids (aa) added to the N-terminus of IE1. In addition, it was found that the leader sequence of *ie0* contains a 4-aa minicistron. To functionally characterize IE0, *ie0* cDNAs were expressed under control of either the *ie1* or the *ie0* promoter. Unexpectedly, examination of *ie0* translation products revealed that the predominant product from *ie0* mRNAs was not IE0, but IE1. Mutation analysis showed that IE1 translation was preferentially initiated from either of two AUGs found in the first 15 nucleotides (nt) of the *ie1* ORF that are internal to the *ie0* ORF. It is unknown whether the internal translation initiation occurs via a leaky scanning mechanism or by an internal ribosomal entry site. Transactivation analysis with constructs that had point mutations in the *ie1* AUGs and were translated only as IE0 revealed that OpMNPV IE0 is a 14- to 15-fold stronger transactivator than IE1. IE0 was also shown to be autoregulatory and to transactivate early genes in an enhancer-independent or -dependent manner. These results suggest that differential expression of baculovirus early genes can be obtained by coexpression of IE0 and IE1 in infected cells, which may permit subtle regulation of specific sets of viral genes.

Key Words: baculovirus; OpMNPV; AcMNPV; *ie1; ie0;* transcription factor.

INTRODUCTION

The baculovirus *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) is a large double-stranded DNA virus that is transcribed and replicates in the nucleus of infected cells. Transcription is temporally coordinated and occurs in three phases: early, late, and very late. Critical to this cascade of gene expression is the early gene *ie1.*

IE1 has multiple essential roles during the baculovirus infection cycle. Analyses of OpMNPV IE1 and the closely related *Autographa californica* MNPV (AcMNPV) IE1 have shown that these functions include transactivation of early and possibly late genes by both enhancer-dependent and -independent mechanisms (Choi and Guarino, 1995b; Guarino and Summers, 1986, 1988; Passarelli and Miller, 1993; Rodems and Friesen, 1995; Theilmann and Stewart, 1991). Using transient assays, IE1 has also been shown to be essential for viral DNA replication (Ahrens and Rohrmann, 1995; Kool *et al.,* 1994). IE1 is a modular transcription transactivator that consists of a number of domains. The N-terminal 135 amino acids (aa) have been shown to be an acidic activation domain that can be functionally replaced by the archetype acidic activation domain from the herpes simplex virus protein VP16 (Forsythe *et al.,* 1998). The AcMNPV IE1 N-terminal domain has also been shown to be an acidic activation domain when placed in heterologous expression systems (Rodems *et al.,* 1997; Slack and Blissard, 1997). In addition, the AcMNPV IE1 C-terminal domains have been shown to be involved in homodimer formation and DNA binding to enhancer regions (Olson *et al.,* 2001).

The *ie1* gene is unique as it is the only baculovirus gene that is known to be spliced to produce a second transcript that is called *ie0.* The OpMNPV *ie1* transcript is expressed as a 1.7-kb mRNA from 0 to 72 h postinfection (p.i.) which increases in steady state levels up to very late times p.i. (Theilmann and Stewart, 1991). The spliced OpMNPV *ie0* transcript is 1.9 kb and is expressed within 1 h p.i., peaks at 4 h p.i., and then declines but remains detectable even up to very late times p.i. Time course analysis using monoclonal antibodies has shown that OpMNPV IE0 translation follows a similar profile as the transcript; that is, peak levels are exhibited early fol-

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0042-6822/01 $35.00

lowed by a decline, but it remains detectable up to very late times p.i. (Theilmann and Stewart, 1993b).

AcMNPV *ie0* cDNA sequence analysis showed that the splicing event added 188 nucleotides (nt) to the 5′ end of the *ie1* transcript, extending the *ie0* ORF at the N-terminus by 54 aa. From the genomic sequence the OpMNPV IE0 was predicted to contain 35 aa added to the N-terminus (Pearson and Rohrmann, 1997), but this was not confirmed by cDNA analysis. Prior to this investigation there had been no studies on the function of OpMNPV IE0 and whether the N-terminus extension changes the functional properties that are already known for IE1. However, AcMNPV IE0 was functionally analyzed by Kovacs *et al.* (1991), who suggested that IE0 had functional differences relative to IE1. Using the *ie1* promoter to drive expression of *ie0* cDNAs, they reported that AcMNPV IE0 was enhancer dependent for gene activation and was not autoregulatory, indicating that the N-terminus extension altered the functional properties of IE1. Kremer and Knebel-Morsdorf (1998) also showed that IE0 binds to enhancer sequences as oligomers and possibly heterodimers with IE1.

Pearson and Rohrmann (1997) analyzed the function of the IE1 homologue in *Lymantria dispar* MNPV (LdMNPV). Using transient replication and transactivation assays, they showed that only IE0 and not IE1 was active in Ld652Y cells, suggesting that splicing was a requirement for producing a functional transactivator. A function for IE1 was not identified.

In this study we investigated the functionality of OpMNPV IE0 to determine its regulatory role during OpMNPV infection. OpMNPV *ie0* cDNAs were cloned and sequenced to confirm the amino acid sequence and the transcriptional start sites. Sequence analysis confirmed that IE0 contains 35 additional aa added to the N-terminus of IE1. In addition, unlike AcMNPV *ie0,* OpMNPV *ie0* contains a 4-aa minicistron 5′ to the *ie0* ORF. *Ie0* cDNAs were expressed under the control of both the *ie1* and the *ie0* promoters, and the surprising result that both IE1 and IE0 are translated from the *ie0* mRNA was observed. Transient assays showed that IE0 was a significantly stronger transactivator than IE1, could activate gene expression in an enhancer-dependent or -independent manner, and, in addition, was autoregulatory. We also examined the expression of AcMNPV *ie0* cDNAs and observed that both IE0 and IE1 were translated from AcMNPV *ie0* mRNAs. Our results indicate that IE0 differs significantly in function from IE1. However, the *ie0* mRNA has novel translational properties that favor translation from internal start codons that results in *ie0* mRNAs mainly producing IE1 and not IE0.

RESULTS

To confirm the sequence and splicing of *ie0* (Fig. 1a) and to generate constructs to express IE0, 5′ rapid amplification of cDNA ends (RACE) was performed and *ie0* cDNAs were cloned and sequenced. Eight full-length clones were sequenced and the transcription start sites identified. From the sequence around the transcriptional consensus start site, ATCAGT, two clones initiated from the first A, two from the C, and four from the second A (Fig. 1b). The sequence also revealed that downstream of the transcriptional start site the *ie0* mRNA contained a minicistron encoding four amino acids. It has been shown for two other baculovirus genes that minicistrons can down regulate translation (Chang and Blissard, 1997; Guarino and Smith, 1990). The *ie0* ORF shows that the predicted IE0 sequence contains 35 amino acids added to the N-terminus of the IE1 protein, of which 23 come from *exon0* (Fig. 1a) and 12 come from the nontranslated leader sequence of *ie1.* This agrees with the sequence predicted by Pearson and Rohrmann (1997).

To express IE0 for functional studies in comparison to IE1, we placed the *ie0* cDNA under control of the *ie1* promoter (p*ie1*-IE0; Fig. 2a). To confirm expression of IE0, the *ie1* expression plasmid, p*ie1*-IE1 (Fig. 2a) and p*ie1*-IE0 were transfected into Ld652Y cells and analyzed by Western blot for expression of IE1 and IE0, respectively (Fig. 3). The transfections were done in duplicate with one set being dephosphorylated, as previously described, to allow for the resolution of single bands (Choi and Guarino, 1995b; Slack and Blissard, 1997). The plasmid p*ie1*-IE1 gave the predicted IE1 band (approximately 64.2 kDa). Surprisingly, plasmid p*ie1*-IE0 did not yield the expected IE0 band. Instead, two bands were detected, corresponding to what appeared to be predominately IE1 and a significantly lower level of a second higher molecular weight band that corresponded to the expected size of IE0 (approximately 68.0 kDa).

This result suggested that translation of *ie0* mRNA expressed from p*ie1*-IE0 was initiating primarily downstream from the start codon at the internal AUG (1° ATG; Fig. 1b), which is the start codon of IE1. The correct transcriptional initiation of *ie0* mRNAs was confirmed by primer extension (data not shown). Functional analysis of IE0 would not be possible if the plasmid produced primarily IE1. Therefore, to confirm that the IE1 band in the p*ie1*-IE0-transfected cells was initiating from the IE1 translational start site, we constructed *ie1* and *ie0* plasmids, p*ie1*-IE1(1° ATG-) and p*ie1*-IE0(1° ATG-), that had point mutations in the IE1 start codon (Fig. 2a). The proteins produced by these constructs were very similar to the proteins expressed from the original plasmids (Fig. 4). The results showed that p*ie1*-IE0(1° ATG-) again produces primarily IE1, as does p*ie1*-IE1(1° ATG-). This result suggested that mutation of 1° ATG results in translation initiating nine bases downstream from the *ie1* 2° ATG (2° ATG; Fig. 1b), which if utilized would produce an IE1 that was three aa smaller.

To confirm that translation from the IE1 and IE0 plasmids was occurring primarily at the 1° or 2° ATGs we

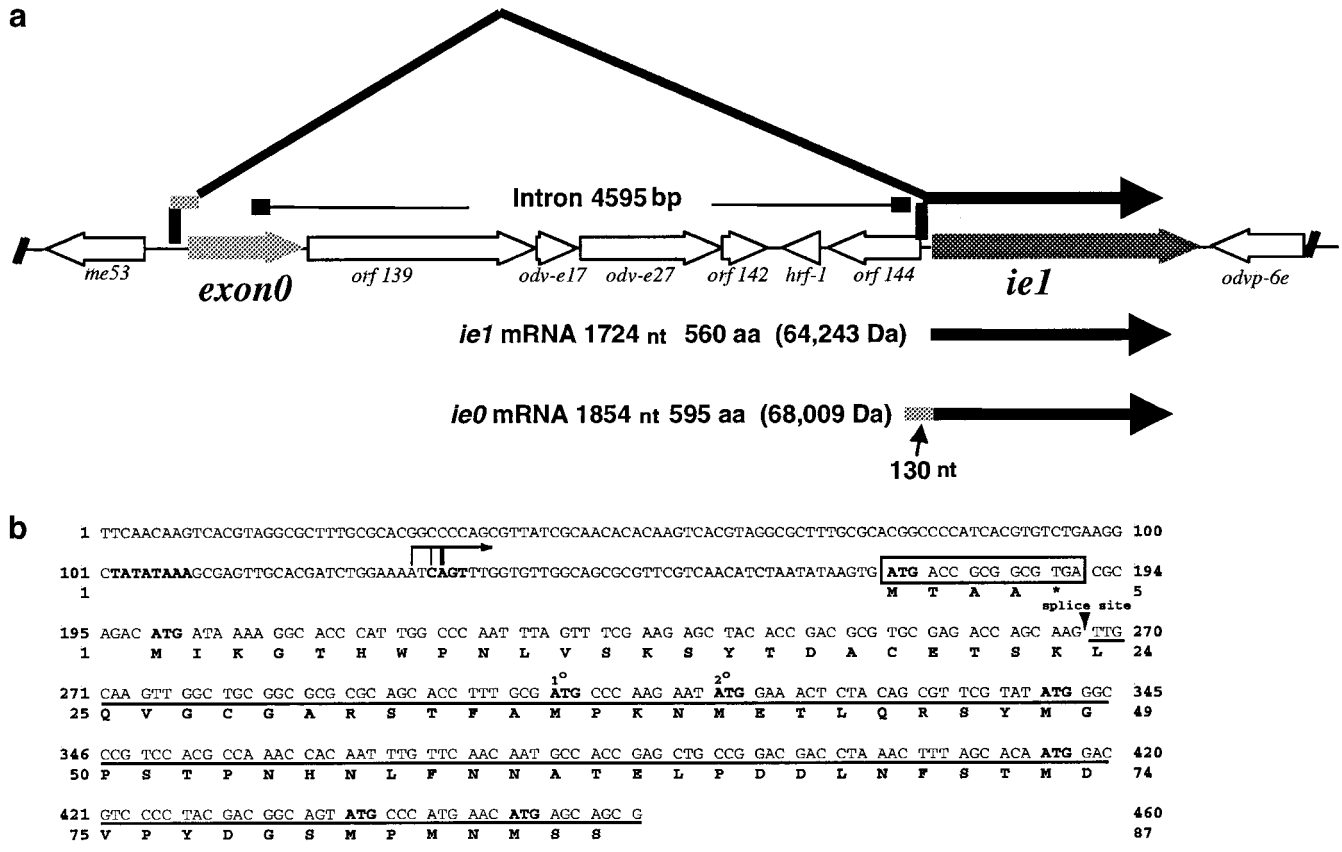

FIG. 1. Map of *exon0,* the *ie1* gene region, and the sequence of *ie0* cDNAs. (a) Schematic map of the OpMNPV genome, from the 5′ end of *me53* to the 3′ end of *odvp-6*e, showing the transcription and splicing pattern of *ie1* and *ie0.* Names of the genes in this region are shown below the ORF arrows. Transcriptional start sites are indicated by vertical black lines. (b) Genomic and *ie0* cDNA sequence. Transcriptional start sites are indicated by vertical black lines and the splice site is indicated by the arrowhead. The four-amino-acid minicistron in the 5′ end of the *ie0* mRNA is boxed 7 bp upstream of the *ie0* start codon. The sequences that originate from the *ie1* gene are underlined. The amino acid sequence of both the minicistron and IE0 is shown below the nucleic acid sequence. The first and second methionine codons of IE1 are labeled 1° and 2°, respectively.

generated two additional IE1 constructs that had mutations in the 2° ATG (p*ie1*-IE1(2° ATG-)) and in both IE1 ATGs (p*ie1*-IE1(2XATG-)). An IE0 plasmid that contained mutations in both of the IE1 ATGs (p*ie1*-IE0(2XATG-); Fig. 2a) was also constructed. Mutation of the *ie1* 2° ATG resulted in an IE1 protein similar in size to that observed when the 1° ATG was mutated (Fig. 4). This shows that the 1° or the 2′ ATG of *ie1* can be used equally well for translation initiation. Mutation of both *ie1* ATGs results in no full-length protein, but smaller translation products the size of which would agree with the third and fourth IE1 ATGs being utilized were observed (see Fig. 1). Mutation of both ATGs in the IE0 expression plasmid did, however, result in IE0 translation and eliminated full-length IE1 translation, but there was translation of IE1 products of less than 66 kDa. The faint band at the size of IE1 in the p*ie1*-IE0(2XATG-) lane was contamination from the IE1 band in the adjacent p*ie1*-IE0(1XATG-) lane.

The set of plasmids using the *ie1* promoter to drive expression of IE1 and IE0 was unable to produce only IE0. To determine whether the promoter and the upstream IE0 UTR would affect production and translation of IE0, a plasmid was constructed in which IE0 expression was placed under the control of the *ie0* promoter (p*ie0*-IE0; Fig. 2b). The resulting *ie0* mRNA contained the minicistron that was identified by sequencing of the IE0 cDNAs (Fig. 1b). In addition, similar to our generation of the *ie1* promoter constructs, we generated a series of plasmids that mutated the 1°, 2°, and both ATGs (p*ie0*-IE0, p*ie0*-IE0(1° ATG-), p*ie0*-IE0(2° ATG-), p*ie0*-IE0(2XATG-), respectively). Each of these plasmids was transfected into Ld652Y cells, and the phosphorylated and dephosphorylated translation products were analyzed by Western blot (Fig. 5). Translation of the p*ie0*-IE0 again resulted in both IE1 and IE0 being produced. However, the level of IE0 was slightly greater relative to that of IE1 when the *ie0* promoter was used, but the predominant translation product remained IE1 (compare p*ie0*-IE0 +CIP (Fig. 5) and p*ie1*-IE0 +CIP (Fig. 4)). The same result was observed when either the 1° or the 2° *ie1* ATG was mutated (p*ie0*-IE0(1° ATG-), p*ie0*-IE0(2° ATG-); Fig. 5). However, mutation of both ATGs did result in p*ie0*-IE0(2XATG-) producing only IE0. This construct

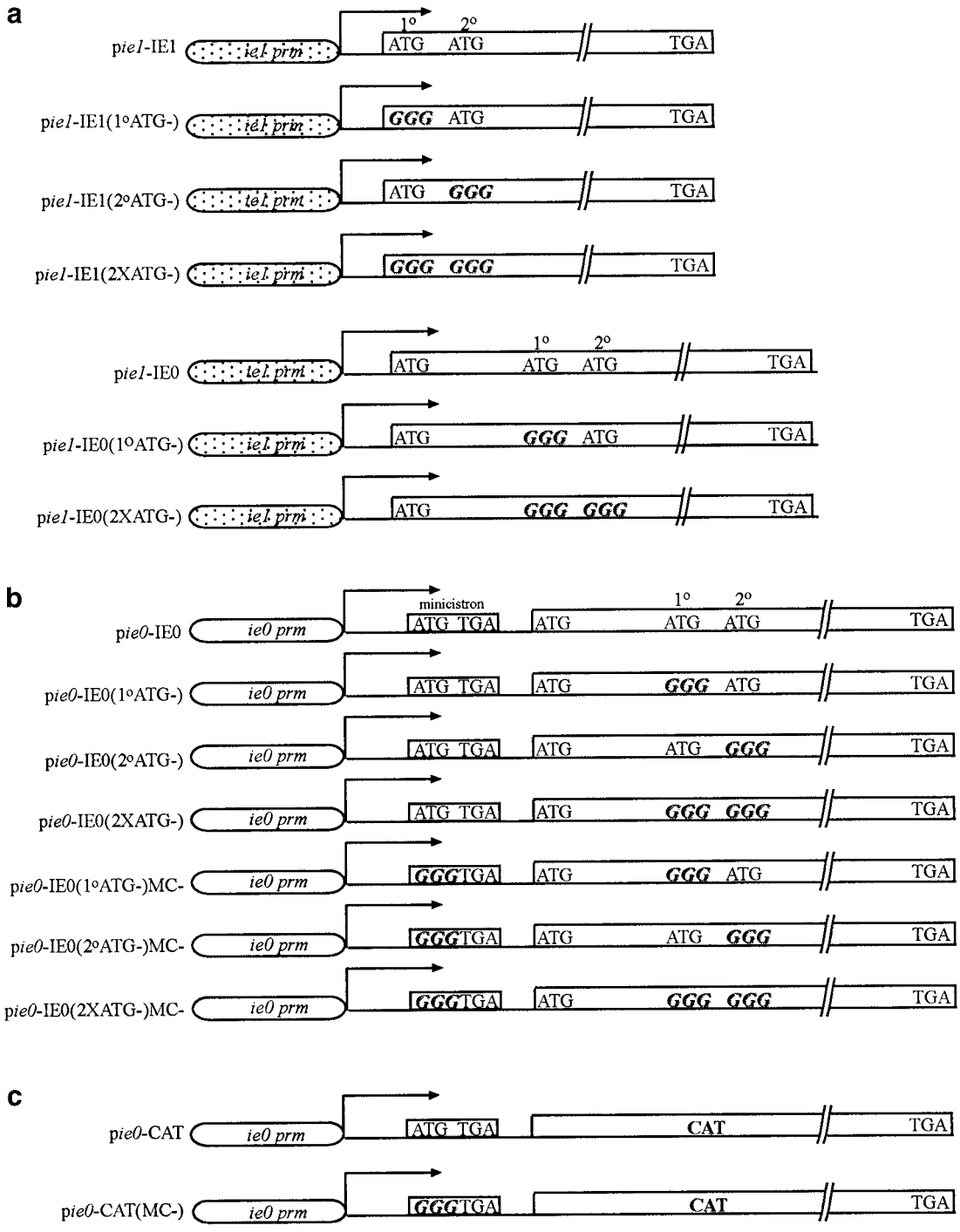

FIG. 2. Schematic diagram of the IE0 and IE1 expression constructs and the CAT reporter constructs. (a) IE0 and IE1 expression constructs using the OpMNPV *ie1* promoter. (b) IE0 and IE1 expression constructs using the OpMNPV *ie0* promoter. The name of the plasmid is shown on the left, and the promoter (*prm*) used to drive expression of each of the proteins is indicated. 1° and 2° refer to the first and second ATG of IE1, respectively (see Fig. 1b). Mutations in the methionine codons are shown in bold. (c) *Ie0* promoter reporter constructs, with a minicistron (p*ie0*-CAT) or without a minicistron (p*ie0*-CAT(MC-)).

would therefore allow the analysis of the function of IE0 in the absence of IE1.

To further analyze the role of the minicistron in IE0 translation we mutated the minicistron ATG in each of the *ie0* expression plasmids (Fig. 2b). Each of these plasmids was transfected into Ld652Y cells and the phosphorylated and dephosphorylated translation products were analyzed by Western blot (Fig. 6). For IE0 production, similar results were observed for the minicistron minus plasmids; that is, only the plasmid with both ATGs mutated (p*ie0*-IE0(2XATG-)(MC-)) translated predominately IE0. The MC- plasmids did, however, produce

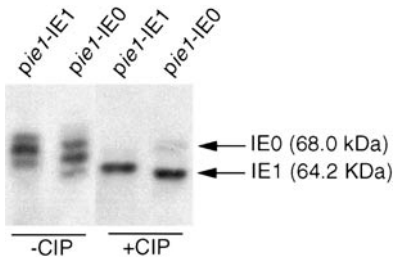

FIG. 3. Western blot analysis of IE0 and IE1 expressed from p*ie1*-IE*1* and p*ie1*-IE0. Ld652Y cells were transfected with either p*ie1*-IE*1* or p*ie1*-IE0 (Fig. 2a). Duplicate protein samples were harvested, one set was dephosphorylated (+CIP), and the other set was left untreated (−CIP). Samples were separated by 7.5% SDS–PAGE, transferred to membranes, and probed with a IE1 monoclonal antibody (Theilmann and Stewart, 1991). The locations of the IE0 and IE1 bands for the +CIP samples are shown on the right.

more IE0 relative to IE1 than the MC+ plasmids, suggesting that the minicistron inhibited translation of downstream ATGs as had been previously reported for other baculovirus genes (Chang and Blissard, 1997; Guarino and Smith, 1990). Interestingly, the minicistron mutation p*ie0*-IE0(2°XATG-)(MC-) resulted in the translation of some significantly smaller proteins that were recognized by the IE1 monoclonal antibody. The origin of these small proteins is unknown. However, they cannot be translation products initiated downstream of the fourth IE1 ATG, as the monoclonal antibody used in these Western blots recognizes an epitope between IE1 aa residues 60 and 80 (unpublished data) and therefore will not bind to N-terminal deletions smaller than approximately 59 kDa. It is also possible that these smaller bands are some form of degradation product. These results indicate that inactivation of the minicistron slightly increased the ratio of IE0 to IE1 from *ie0* expression plasmids, but did not stop translation from downstream IE1 AUGs.

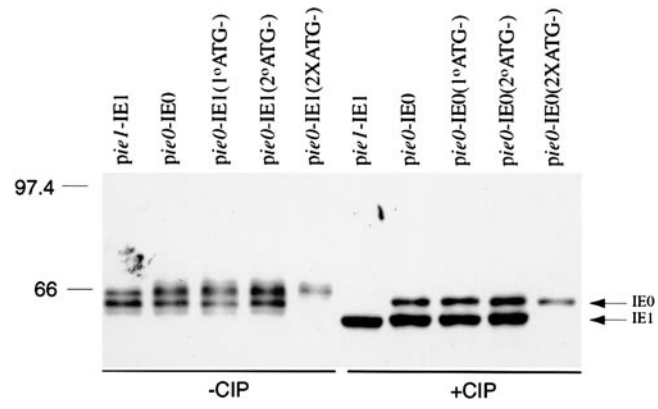

FIG. 5. Western blot analysis of IE0 and IE1 products expressed form mutated and nonmutated plasmids under the control of the *ie0* promoter with an intact minicistron in transfected Ld652Y cells. The name of the construct (Fig. 2) used is indicated above each lane. Samples were prepared as described in the legend to Fig. 3. The locations of the IE0 and IE1 bands for the +CIP samples are indicated on the right. Molecular weight size markers (kDa) are indicated on the left.

Transactivation analysis of IE0

Analysis of IE0 translation products has shown that *ie0* mRNAs produce predominately IE1. However, the mutant constructs we generated provided the tools for comparing the function of IE0 relative to IE1. Using the OpMNPV enhancer containing reporter plasmid p39CAT-E3 (Theilmann and Stewart, 1992b) we tested the ability of IE0 and IE1, as expressed from our various constructs, to activate gene expression. Figure 7 compares the abilities of p*ie0*-IE0, p*ie0*-IE0(1° ATG-), p*ie0*-IE0(2° ATG-), and p*ie0*-IE0(2XATG-) to transactivate p39CAT-E3 relative to p*ie1*-IE1. The results showed that all constructs were able to transactivate the reporter gene. Therefore, IE0

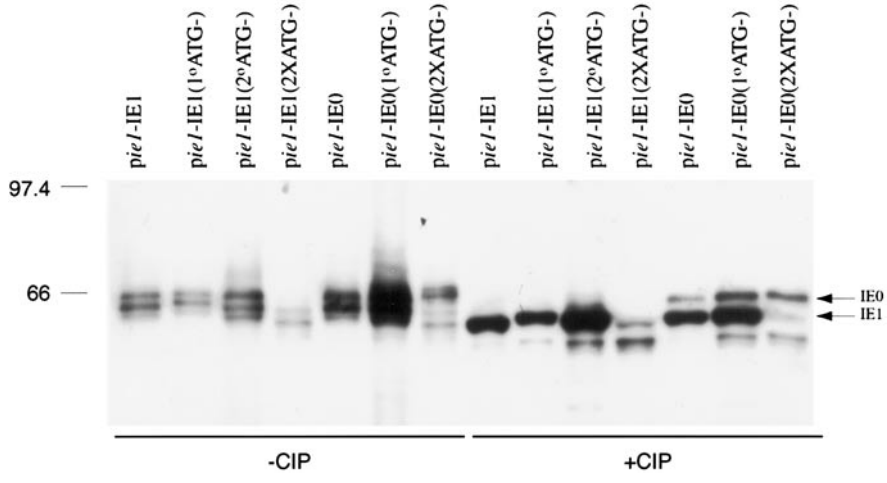

FIG. 4. Western blot analysis of IE0 and IE1 translation products expressed from mutated and nonmutated plasmids under the control of the *ie1* promoter. The name of the construct (Fig. 2) used is indicated above each lane. Samples were prepared as described in the legend to Fig. 3. The locations of the IE0 and IE1 bands from the mutated constructs for the +CIP samples are shown on the right. Molecular weight size markers (kDa) are indicated on the left.

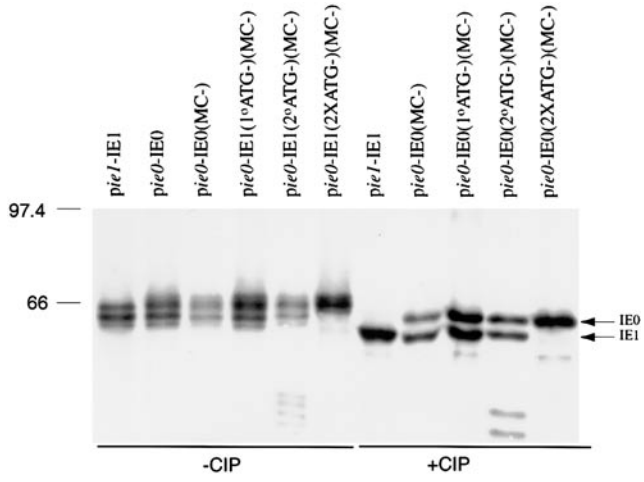

FIG. 6. Analysis of the role of the *ie0* minicistron on IE0 translation. Western blot analysis of IE0 and IE1 products expressed form mutated and nonmutated plasmids under the control of the *ie0* promoter with a mutated minicistron in transfected Ld652Y cells. The name of the construct (Fig. 2) used is indicated above each lane. Samples were prepared as described in the legend to Fig. 3. The locations of the IE0 and IE1 bands are indicated on the right. Molecular weight size markers (kDa) are indicated on the left.

expressed from p*ie0*-IE0(2XATG-), which has the second and third methionines mutated to glycines, is a functional transactivator.

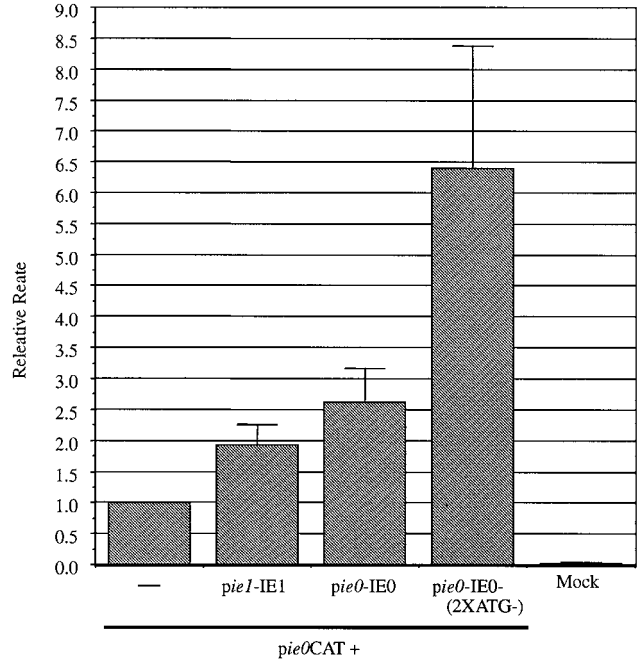

FIG. 8. Autoregulation analysis of *ie0*. The reporter plasmid p*ie0*-CAT (Fig. 2c) was cotransfected into Ld652Y cells with p*ie1*-IE1, p*ie0*-IE0, and p*ie0*-IE0(2XATG-). Values given are reported relative to the reporter plasmid alone (—), which was given the value of 1.0. Error bars represent the standard error.

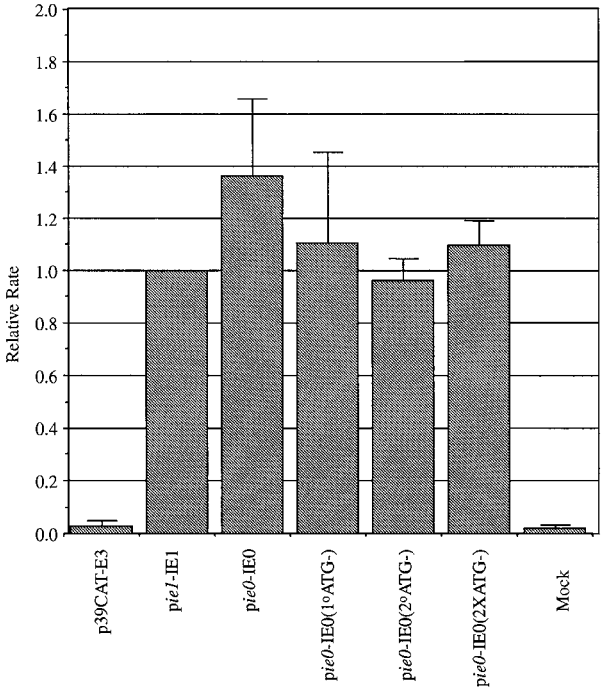

FIG. 7. Transactivation analysis of p39CAT-E3 by IE1 and IE0. The reporter plasmid p39CAT-E3, which contains the CAT gene under the control of the AcMNPV p39 promoter with an OpMNPV enhancer 3′ (Theilmann and Stewart, 1992b), was cotransfected in Ld652Y cells with plasmids expressing IE1, IE0, or IE0 with the 1° or 2° ATG mutated (Fig. 2). Values given are reported relative to p*ie1*-IE1, which was given the value of 1.0. Error bars represent the standard error.

Kovacs *et al.* (1991) reported that the *ie0* gene of AcMNPV was not autoregulatory like *ie1.* To determine whether OpMNPV IE0 had similar properties, we constructed an *ie0* reporter plasmid, p*ie0*-CAT (Fig. 2c), that replaced the *ie0* ORF with the reporter gene CAT. p*ie0*-CAT was cotransfected with p*ie1*-IE0, p*ie0*-IE0, and p*ie0*-IE0(2XATG-). The *ie0* promoter itself was highly active (Fig. 8); however, cotransfection with p*ie1*-IE1, which expresses only IE1 (Fig. 4) caused a 2-fold activation. The plasmid p*ie0*-IE0, which expresses mainly IE1 and a small amount of IE0, caused a 2.5-fold increase in p*ie0*-CAT expression. Expression of only IE0 from p*ie1*-IE0(2XATG-) caused the most significant activation (nearly 6.5-fold) of p*ie0*-CAT. These results show that, unlike the findings reported for the *ie0* gene of AcMNPV, the OpMNPV *ie0* gene is autoregulatory.

We have previously shown that the *ie1* promoter, in addition to being autoregulated by IE1, is also transactivated by IE2 (Theilmann and Stewart, 1992a). To determine whether the *ie0* promoter is similarly regulated, we performed transient assays that transactivated p*ie0*-CAT with IE2. In addition, we made a second *ie0* reporter plasmid that mutated the minicistron ATG (p*ie0*-CAT(MC-); Fig. 2b) to determine whether loss of the minicistron affected expression of the downstream ORF (CAT). As shown in Fig. 9, IE2 was observed to be a strong transactivator of p*ie0*-CAT, causing a sevenfold activation. Mutation of the minicistron also enabled

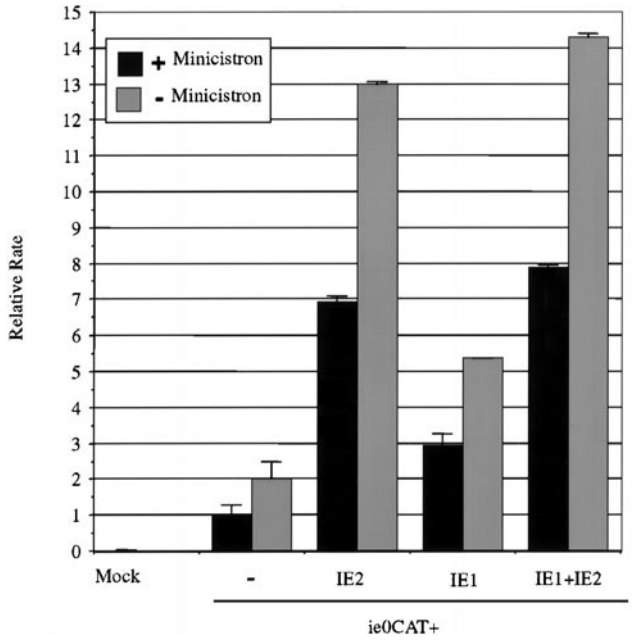

FIG. 9. Analysis of IE1 and IE2 transactivation of the *ie0* promoter and effect of the minicistron on reporter gene expression. The reporter plasmid p*ie0*-CAT (Fig. 2c) was cotransfected into Ld652Y cells with constructs expressing IE1 and IE2, as indicated. Values given are reported relative to the reporter plasmid alone (—), which was given the value of 1.0. Error bars represent the standard error.

greater p*ie0*-CAT activation by IE1. No significant synergism was observed when both IE1 and IE2 were cotransfected with the *ie0*-CAT reporter plasmids. Therefore, mutation of the minicistron permits nearly a twofold increase in gene expression with or without the transactivators IE1 and IE2. These data agree with those depicted in Fig. 6, which showed that mutation of the minicistron increased expression of IE0, as determined by Western blot. These results support the interpretation that the *ie0* minicistron down regulates IE0 expression.

To further investigate the function of IE0 and its ability to activate baculovirus promoters, we analyzed four additional reporter constructs, AcMNPV p39CAT, OpMNPV *ie1*CAT, OpMNPV *opep2*CAT, and OpMNPV p*64*CAT-166 (Blissard and Rohrmann, 1991; Carson *et al.,* 1988; Shippam *et al.,* 2001; Theilmann and Stewart, 1991). These four reporter constructs were transfected into Ld652Y cells separately or with p*ie1*-IE1, p*ie0*-IE0, or p*ie0*-IE0(2XATG-), which will produce IE1, IE1 and IE0, and IE0, respectively. Analysis of p39CAT expression showed that p*ie0*-IE0 or p*ie0*-IE0(2XATG-) caused significantly greater activation than p*ie1*-IE1 (Fig. 10a). This result is intriguing, since the same reporter construct with an enhancer at the 3′ end of the gene (Fig. 7) showed no difference in gene activation by IE1 and IE0. OpMNPV IE0 is therefore not enhancer dependent and appears to be more active than IE1 on enhancerless promoters.

Activation of p*ie1*-CAT and p*64*CAT-166 revealed even greater differences between IE1 and IE0. p*ie1*-CAT was activated 6.6-fold by IE1, 14-fold by IE1/IE0, and 35-fold by IE0(2XATG-) (Fig. 10a). Similarly, p*64*CAT-166 was activated most strongly by IE0(2XATG-), causing a 16-fold increase, whereas IE1 and IE1/IE0 caused a 3.5- and a 4.4-fold increase, respectively. Figure 10b shows a Western blot analysis of the levels of IE1 and IE0 produced in the transfected cells of p*ie1*-CAT and p*64*CAT-166. Densiometric analysis showed that the p*ie0*-IE0(2XATG-)-transfected cells produced approximately one-third the level of protein of p*ie1*-IE1-transfected cells. This indicates that the specific activity of IE0 for transactivating specific promoters is 14- to 15-fold higher than that of IE1; thus, IE0 is a considerably stronger transactivator than IE1.

The OpMNPV p*opep*CAT construct has been shown to not be activated by IE1 (Shippam *et al.,* 1997). Cotransfection with IE0-expressing constructs produced the

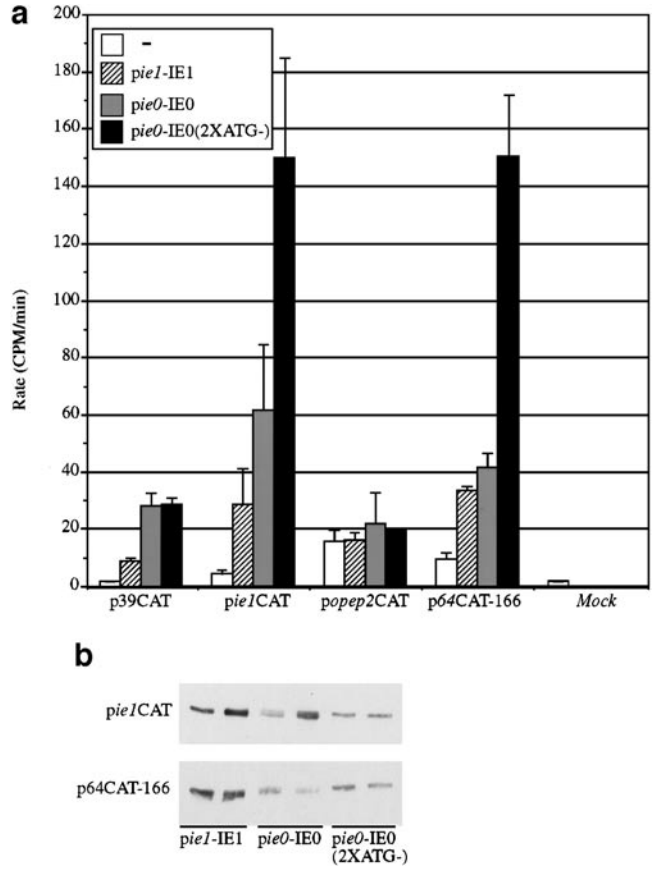

FIG. 10. Transactivation of selected enhancerless early promoters by p*ie1*-IE0, p*ie0*-IE0, and p*ie0*-IE0(2XATG-). (a) The reporter plasmids p39CAT, p*ie1*CAT, p*opep2*-CAT, and p*64*CAT-166 were cotransfected with p*ie1*-IE1, p*ie0*-IE0, and p*ie0*-IE0(2XATG-), and the levels of CAT expression were determined. Error bars represent the standard error. (b) Western blot analysis of the cells transfected in (a) with p*ie1*CAT and p*64*CAT-166. Samples were separated by 12% SDS–PAGE, transferred to membranes, and probed with an IE1 monoclonal antibody (Theilmann and Stewart, 1991). Under these conditions IE1 and IE0 comigrate and are detected as a single band.

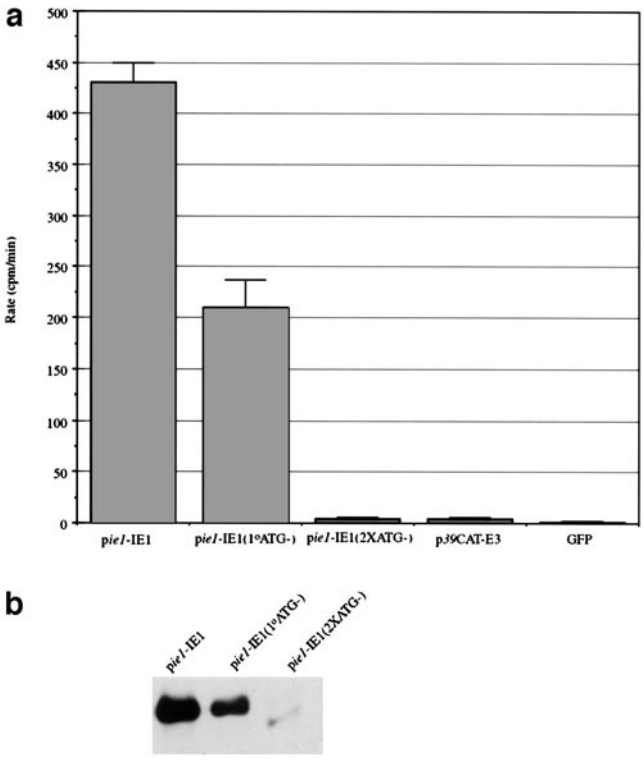

FIG. 11. Transactivation capability of IE1 constructs initiating from different start codons. (a) The reporter plasmid p39CAT-E3 was cotransfected with p*ie1*-IE1, p*ie1*-IE1(1° ATG-), or p*ie1*-IE1(2XATG-), which represent wild-type IE1 or IE1 with the 1° and 2° methionines mutated, respectively (see Fig. 2a). Error bars represent the standard error. (b) Western blot analysis of the cells transfected in (a) with p*ie1*-IE1, p*ie1*-IE1(1° ATG-), or p*ie1*-IE1(2XATG-).

same results. That is, IE0 did not activate the *opep2* promoter, which suggests that even though IE0 appears to be a stronger transactivator, the specificities for the promoters tested in this study were not altered by the addition of the IE0 domain to the IE1 protein.

In Figs. 4 and 5 we showed that IE1 produced from *ie0* transcripts initiated from either the 1° or the 2° ATG. Whether an IE1 that initiates from the 2° ATG has the same activity as one initiating from the 1° ATG is unknown. To compare the activities of IE1 initiated from either ATG we cotransfected p39CAT-E3 with p*ie1*-IE1, p*ie1*-IE1(1° ATG-), and p*ie1*-IE1(2° ATG-) and assayed for CAT expression (Fig. 11a). In addition, we analyzed the transfected samples by Western blot for IE1 expression levels (Fig. 11b). The CAT assays showed that p*ie1*-IE1(1° ATG-) had half the level of expression of wild-type IE1. However, Western blots revealed that p*ie1*-IE1(1° ATG-) produced only half as much as p*ie1*-IE1. Therefore, on a per molecule basis, IE1 translated from either the 1° or 2° ATG appears to be equally potent for transactivation of an enhancer-containing reporter construct. We also cotransfected p*ie1*-IE1(2XATG-) with p39CAT-E3 and found that the translation product was inactive. The IE1 translation product from p*ie1*-IE1(2XATG-) was effectively an N-terminal truncation of IE1. These data agrees with a previous study by Forsythe *et al.* (1998), which showed that very small N-terminal deletions of the acidic domain of IE1 caused complete loss of activity.

Our discovery that *ie0* mRNAs were translated to produce both IE0 and IE1 was very surprising. To determine whether this also occurs for other baculovirus *ie0* genes we analyzed IE0 expression from the corresponding AcMNPV gene (Fig. 12). For this analysis we placed both the *ie0* and the *ie1* ORFs of AcMNPV under the control of the OpMNPV *ie2* promoter (Hegedus *et al.,* 1998; Pfeifer *et al.,* 1997) and established stable cell lines expressing both genes. We analyzed the AcMNPV IE0 and IE1 gene products by Western blot, as shown in Fig. 12. The results clearly showed that AcMNPV IE0 was expressed as both IE0 and IE1. Therefore, the AcMNPV *ie0* gene is translated in the same way as the *ie0* gene of OpMNPV, suggesting that this may be a general mechanism of IE0 expression in baculoviruses.

DISCUSSION

Ie0 is the only known baculovirus gene that is spliced. The OpMNPV *ie0* transcript consists of 130 nt from *exon0* which is spliced onto the 5′ end of the *ie1* transcript that initiates 4595 bp downstream. This results in 35 aa being added to the N-terminus of IE1, of which 23 aa are coded for by the upstream exon and 12 aa by the *ie1* 5′-UTR. The objective of this study was to determine what functions IE0 gains by the addition of 35 aa to the N-terminus of IE1. In this study, we showed that expression of OpMNPV *ie0* mRNAs results unexpectedly in the translation of only small amounts of IE0 and high levels of IE1. Mutation analysis showed that *ie1* is internally initiated from either the first or the second AUG of the *ie1* coding sequences. Transactivation analysis of baculovirus early promoters using a construct that only expresses IE0 revealed that IE0 transactivates genes in an enhancer-dependant or -independent manner and is autoregulatory. In addition, our data indicated that IE0 was a significantly (14- to 15-fold) stronger transcriptional activator of specific early genes than IE1. Our data also demonstrated that both IE0 and IE1 were translated from AcMNPV *ie0* cDNAs, suggesting that this may be a common mechanism for IE0–IE1 expression in baculoviruses.

Previous analysis of the AcMNPV *ie0* gene by Kovacs *et al.* (1991) described functional differences between the abilities of IE0 and IE1 to transactivate genes. By expressing AcMNPV IE0 from a plasmid regulated by the AcMNPV *ie0* promoter, they showed that IE0 was enhancer dependent and could not activate the *ie0* promoter. In Fig. 12 we expressed AcMNPV IE0 under the control of the OpMNPV *ie2* promoter and observed that both IE0 and IE1 were expressed in a manner similar to that which we had observed with OpMNPV IE0. In the study by Kovacs *et al.* (1991), the expressed IE0 products

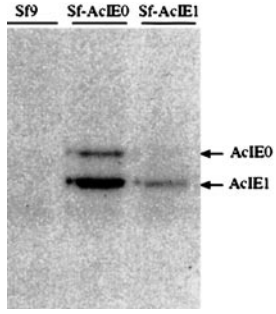

FIG. 12. Analysis of AcMNPV *ie0* translation in stably transformed Sf9 cells. Sf9 cell lines stably transformed with constructs expressing AcMNPV IE0 and IE1 under control of the OpMNPV *ie2* promoter were analyzed by Western blot using a monoclonal antibody specific to AcMNPV IE1. Sf9, mock; Sf-AcIE0, Sf9 cells transformed with a construct expressing AcMNPV IE0; Sf-AcIE0, Sf9 cells transformed with a construct expressing AcMNPV IE1.

were not analyzed by Western blots; therefore, based on our results, it is possible that the AcMNPV IE0 activity reported was actually due to a mixture of IE1 and IE0. However, by comparing similar OpMNPV and AcMNPV constructs we observed that OpMNPV IE0 has properties significantly different from those reported for AcMNPV IE0 and may reflect species differences. That is, OpMNPV IE0 is not enhancer dependent, can transactivate the *ie0* promoter, and appears to be a significantly more potent transactivator than IE1.

Pearson and Rohrmann (1997) analyzed the activity of the IE0 and IE1 genes of LdMNPV. Similar to our findings for OpMNPV IE0, the observations of that study were that LdMNPV IE0 was able to transactivate the AcMNPV *p39* promoter with or without a *cis*-linked LdMNPV or AcMNPV enhancer region. Expression from the enhancer-containing clones was, however, significantly higher than that from the enhancerless constructs. Interestingly, the LdMNPV *ie1* gene was found to be totally inactive in both transactivation assays and transient replication assays, which suggested that splicing was required to obtain functional IE1. It is possible, based on the results of this study, that both IE1 and IE0 are translated from the LdMNPV *ie0* transcripts.

Our experiments show that IE0 is a stronger transactivator than IE1. The 35 aa that are added to the N-terminus of IE1 to produce IE0 have no identifiable motifs and are not highly conserved between baculoviruses (Pearson and Rohrmann, 1997). The N-terminus of OpMNPV IE1 is an acidic activation domain (Forsythe *et al.,* 1998), but the 35 additional aa of IE0 do not maintain the acidic profile, suggesting that it is not just a domain extension. It is possible that the 35 aa permit a more efficient recruitment of the RNA Po III complex directly or via cofactors (Lemon and Tjian, 2000). Alternatively, IE0 may bind promoters or enhancer regions more efficiently than IE1, but further experimentation will have to be done to determine how IE0 mediates a stronger gene activation than IE1.

In the context of an infected cell, IE0 and IE1 are always coexpressed and therefore mediate their functions in a mixed environment. AcMNPV IE1 binds to enhancer regions as a homodimer through a helix–loop–helix-like domain located in the C-terminal (residue 543 to residue 568) region (Choi and Guarino, 1995a, b; Olson *et al.,* 2001; Rodems and Friesen, 1995). Kremer and Knebel-Morsdorf (1998) have provided evidence that homodimers and heterodimers of IE0 and IE1 can form, producing a tripartite complex in band shift assays using the IE1 binding region of the AcMNPV *he65* promoter. Therefore, in infected cells IE0-IE0, IE0-IE1, and IE1-IE1, dimers could all be present and activate promoters to various levels. The results shown in Figs. 7, 8, and 10 indicate that a gradation of gene activation occurs in transfected cells depending on the presence of IE1 alone, IE1-IE0, or IE0 alone. Therefore, it is possible that each type of dimer could have different binding affinities for different promoters, activating genes to varying levels, which would provide a subtle mechanism for regulating different promoters during infection. At late times postinfection, since IE0 is expressed at significantly lower levels (Choi and Guarino, 1995b; Kremer and Knebel-Morsdorf, 1998; Theilmann and Stewart, 1993a), promoters which are preferentially activated by IE1 homodimers would be predominately expressed over promoters activated by IE0-IE1 or IE0-IE0 dimers. Functional changes to viral transactivators by the addition of small domains through splicing have been aptly demonstrated for the extensively studied adenovirus E1A *12S* and *13S* genes. The *13S* E1A gene product only differs from *12S* by 46 aa in the transactivation domain, yet this has been shown to change its activation function and its promoter specificity (Liu and Green, 1994).

Translation of IE0

The most unexpected result of this study was that translation of the OpMNPV *ie0* transcript was primarily initiated at the third or fourth AUG downstream of the 5′ end of the mRNA. Mutation of the minicistron AUG did increase the ratio of IE0 to IE1 (Fig. 6), which suggests that the minicistron down regulates IE0 translation. Chang and Blissard (1997) did an extensive analysis of the minicistron of the OpMNPV gp64 and also showed that it down regulated translation from the downstream ORF.

Translation of *ie0* mRNAs is preferentially initiated at the third or fourth AUG, which are located 194 and 206 nt from the 5′ end of the *ie0* mRNA, respectively. The exact mechanism by which this occurs is not yet known but is likely due to either leaky scanning (Chang *et al.,* 1999) or an internal translation initiation, possibly mediated by an internal ribosomal entry site (IRES). There is no prece-

dent for internal initiation in baculoviruses, although an insect IRES has been potentially identified in Drosophila (Kozak, 2001; Ye *et al.,* 1997). Leaky scanning does occur on the gp64 promoter, but in terms of the translation start site context, the AUGs of the minicistron and the IE0 ORF would be predicted to have the most efficient translational start sites. This is based on either Chang *et al.* (1999) (5′-NNNNNAUGAa/c/gN-3′) or the Kozak consensus sequence (PuNNAUGPu) (Kozak, 1989). Therefore, the predominant downstream IE1 translation start sites would not be expected to be the most efficiently utilized (Figs. 5 and 6).

It is very surprising that the *ie0* transcripts are translated predominately as IE1, since the virus produces *ie1* transcripts very efficiently and to very high levels (Theilmann and Stewart, 1991). This would suggest that maintaining IE0 at low levels is a priority for OpMNPV during normal virus infections. It is tempting to speculate that IE0 is required for functions other than early gene transactivation, such as viral DNA replication, where high levels of IE0 may be deleterious. The functional differences that we have observed between IE0 and IE1 add another level of complexity to baculovirus gene expression and replication.

MATERIALS AND METHODS

Viruses and cells

L. dispar (Ld652Y) cells were maintained in TC100 medium as described (Summers and Smith, 1987). OpMNPV was propagated in Ld652Y cells as previously described (Quant-Russell *et al.,* 1987).

5′ RACE

The rapid amplification of cDNA ends strategy (Frohman *et al.,* 1988; Ohara *et al.,* 1989) was used to obtain clones containing the *ie0* gene of OpMNPV. 5′ RACE reactions of total RNA from mock- or virus-infected Ld652Y cells were run in parallel. Reverse transcription was carried out by combining 5 μg of total RNA from mock- or virus-infected Ld652Y cells with 10 pmol of GSP-1, a negative-sense OpMNPV *ie1* gene-specific oligonucleotide, 5′-TGCGACTCAACATTATGG-3′. Synthesis of the second strand, tailed cDNA, was primed with the positive-sense oligonucleotide XBEdT, 5′-CTCGAGGGATCCGAATTCTTTTTTTTTTTTTTTT-3′. The *ie1* and *ie0* 5′ cDNAs were amplified with two oligonucleotides, positive-sense XBE (*Xho*I–*Bam*HI–*Eco*RI), 5′-CTCGAGGGATCCGAATTC-3′, and the *ie1*-specific, negative-sense IE1-4if (5′-GATTCTAGACGTCGTTACACAGGTTCACG-3′; *Xba*I site is underlined). Two major PCR amplification products of 1054 and 919 bp corresponding to the 5′ clones of *ie0* and *ie1,* respectively, were purified, digested with *Bam*HI and *Xba*I, and cloned into the plasmid vector pBS+ (Stratagene).

Plasmid constructs

To construct p*ie1*-IE1, the *ie1* ORF and 3′ sequences from the plasmid Op47SalI-IE1 (Theilmann and Stewart, 1993b) were PCR amplified using two primers, p133 (5′ GCGGATCCGCGATGCCCAAGA 3′) and the M13 reverse primer (5′ AACAGCTATGACCATG 3′). The amplified product, containing a primer-generated *Hin*dIII site 122 bp downstream of the IE1 ORF and a *Bam*HI site at the 5′ end, was subcloned into the *Bam*HI–*Hin*dIII site of the plasmid OpIE1*Bam*(ATG-), which contained the 1.7-kb p*ie1* promoter. The resulting construct contained an insertion of a 10-bp sequence 4 bp upstream of the *ie1* ORF containing a *Bam*HI site.

To construct p*ie1*-IE0, the C-terminal 960 bp of *ie0* from a cDNA clone containing the *ie0* 5′ sequences was PCR amplified, using the two primers p135 (5′ CGGGATCCGACATGATAAAAGGCACCCATTG 3′), containing a *Bam*HI site, and pIE1-4if (5′ GATTCTAGACGTCGTTACACAGGTTCACG 3′). The amplified product contained an internal *Eco*NI site that could also be found in the *ie1* ORF. The *Bam*HI–*Eco*NI region was subcloned into the *Bam*HI–*Eco*NI sites of p*ie1*-IE1, and the resulting plasmid was designated p*ie1*-IE0.

The plasmids p*ie0*-IE0, p*ie0*-IE0(1° ATG-), p*ie0*-IE0(2° ATG-), and p*ie0*-IE0(2XATG-) were constructed by amplifying the promoter sequence of IE0 from plasmid Op47*Bgl*1.0 by PCR. Two primers, p156 (5′ TAGGAGCTCCCGTACAACACAATACTGTGCATGT 3′) and p157 (5′ TAGGATCCTGCGTCACGCCGCGGTCAT 3′), were used to create an *Sst*I site at the 5′ end and a *Bam*HI site at the 3′ end of the amplified *ie0* promoter, which contained 356 bp upstream of the *ie0* start codon. The PCR fragment was cut with *Bam*HI and cloned into the vectors p*ie1*-IE0, p*ie1*-IE0(1° ATG-), p*ie1*-IE0(2° ATG-), and p*ie1*-IE0(2XATG-), all of which had the *ie1* promoter removed. The *ie1* promoter was removed by an initial *Eco*RI digestion. The *Eco*RI ends were filled in and made blunt with *Pfu* polymerase, followed by a *Bam*HI digestion, which dropped out the *ie1* promoter.

The plasmid used for expression of the OpMNPV transactivator IE2 was pIE2-E2.3 (Theilmann and Stewart, 1992a). The p64CAT-166 plasmid was constructed by Blissard and Rohrmann (1991).

Site-directed mutagenesis

All site-directed mutagenesis was performed using *Pfu* DNA polymerase (Strategene) by the method described by Fisher and Pei (1997). The plasmid p*ie1*-IE1(1° ATG-) was generated by mutating the 1° ATG to a glycine codon (GGG) using the primers p158 (5′ GGGCCCAAGAATATGGAAACTCTACAGCGTTCGTATA 3′) and p159 (5′ CGCGGATCCCGGCAAAGGTGCT 3′) and using p*ie1*-IE1 as a template.

The plasmid p*ie1*-IE1(2° ATG-) was generated by mu-

tating the 2° ATG to a glycine codon (GGG) using the primers p166 (5′ ATGCCCAAGAATGGGGAAACTCTACAGCGTTCGTATA 3′) and p159 (see above) and using p*ie1*-IE1 as a template.

The plasmid p*ie1*-IE1(2XATG-) was generated by mutating the 1° and 2° ATGs to glycine codons (GGG) using the primers p160 (5′ GGGCCCAAGAATGGGGAAACTCTACAGCGTTCGTATA 3′) and p159 (see above) and using p*ie1*-IE1 as a template.

The plasmid p*ie1*-IE0(1° ATG-) was generated by mutating the 1° ATG to a glycine codon (GGG) using the primers p158 (see above) and p161 (5′ CGCAAAGGTGCTGCGCGCGCCGCAG 3′) and using p*ie1*-IE0 as a template.

The plasmid p*ie1*-IE0(2° ATG-) was generated by mutating the 2° ATG to a glycine codon (GGG) using the primers p166 (see above) and p161 (see above) and using p*ie1*-IE0 as a template.

The plasmid p*ie1*-IE0(2XATG-) was generated by mutating the 1° and 2° ATGs to glycine codons (GGG) using the primers p160 (see above) and p161 (see above) and using p*ie1*-IE0 as a template.

The plasmids p*ie0*-IE0(MC-), p*ie0*-IE0(1° ATG-)(MC-), p*ie0*-IE0(2° ATG-)(MC-), and p*ie0*-IE0(2XATG-)(MC-) were generated by mutating the minicistron ATG to TTG using the primers P168 (5′ GTTGACGAACGCGCTGCCAACACCAAACTG 3′) and P167 (5′ ATCTAATATAAGTGTTGACCGCGGCGTGAC 3′). The templates used were p*ie0*-IE0, p*ie0*-IE0(1° ATG-), p*ie0*-IE0(2° ATG-), and p*ie0*-IE0(2XATG-), respectively.

The reporter plasmid p*ie0*-CAT was constructed by amplifying the *ie0* promoter by PCR (see above). The resulting fragment was subcloned into the pIE1CAT-*Sal* plasmid (Theilmann and Stewart, 1993b), which was digested with *Sst*I and partially digested with *Bam*HI to remove the 634-bp IE1 promoter. This clone was designated p*ie0*CAT. The minicistron was mutated using the primers P167 and P168 (see above) with p*ie0*CAT as a template, which generated the plasmid p*ie0*CAT(MC-).

DNA sequencing and primer extensions

Manual DNA sequencing was carried out using Sequenase version 2.0 (Amersham) as described (Toneguzzo *et al.,* 1988). DNA for use in automated sequencing was purified using the QIAGEN Miniprep kit. Automated sequencing of DNA was carried out using the ABI Prism Dye Terminator Cycle Sequencing Kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems), following the manufacturer's instructions. Primer extensions were performed as previously described by Theilmann and Stewart (1991) using the primer P205 (5′ CATATACGAACGCTGTAGAG 3′), which hybridizes 39 bp downstream of the *ie1* start codon.

Transfections and CAT assays

Liposomes for transfection were made according to Campbell (1995). Liposomes were titered for the optimal liposome/DNA ratio—2 μg DNA in total was transfected for each sample. One microgram of reporter plasmid and 1 μg of transactivator (if appropriate) were transfected for each sample; pBS+ was included in the transfection mixture as required to ensure equivalent quantities of DNA were delivered to each well. To assay for CAT activity, cells were scraped off dishes 48 h posttransfection and pelleted, all media were removed, and the cell pellet was resuspended in 200 μl 250 mM Tris–HCl (pH 7.8). Cells were lysed by freeze-thawing (three cycles) and any cellular deacetylases inactivated by incubation at 65°C for 15 min, with a final short centrifugation to pellet cell debris. Cell extracts were titered to determine the appropriate quantity of extract to use to ensure a linear response in the assay. To assay, an appropriate volume of cell extract (1–50 μl) was combined with 6.25 mM chloramphenicol, 160 mM Tris–HCl (pH 7.8), 3.2 μM acetyl-coenzyme A (Sigma), and 0.025 μCi (125 pmol) [^{3}H]acetyl-coenzyme A (New England Nuclear, CAT Assay Grade) in a total volume of 125 μl (Neumann *et al.,* 1987). Assay rates were normalized to the parent reporter construct for each set of experiments, which was assigned a value of 1 to allow comparison of several experiments. All transfections were repeated a minimum of three times, each in duplicate.

Western blots

Transfected cells were prepared for calf intestinal phosphatase (CIP, Sigma) treatment as described by Slack and Blissard (1997). Proteins were separated using denaturing gel electrophoresis (SDS–PAGE; Laemmli, 1970) and transferred to Immobilon membranes (Millipore) using standard techniques (Sambrook *et al.,* 1989). To detect OpMNPV IE1, blots were incubated with the mouse monoclonal antibody IE1-10 (Theilmann and Stewart, 1993a). Bound antibody was then detected using goat anti-mouse peroxidase-linked antibody (The Jackson Laboratories) and the ECL (Amersham) substrate using the manufacturer's specifications. To detect AcMNPV IE1 blots were incubated with a mouse monoclonal antibody (Ross and Guarino, 1997).

Stable cell lines

Stable cell lines expressing AcMNPV IE1 and IE0 were prepared using the vector p2ZOp2F (Hegedus *et al.,* 1998) and the method described by Pfeifer *et al.* (1997).

ACKNOWLEDGMENTS

The authors thank D'Ann Rochon, Guus Bakkeren, and Jane Theilmann for critical reading and very helpful comments on the manuscript. The plasmid p64CAT-166 and the AcMNPV IE1 monoclonal

antibody were kindly provided by Dr. Gary W. Blissard and Dr. Linda Guarino, respectively. The oligonucleotide XBEdT was a gift from Dr. Aly Karsan. This study was supported in part by a Natural Sciences and Engineering Research Council Research Grant awarded to D.A.T. This article is Contribution No. 2129 from the Pacific Agri-Food Research Centre.

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